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REPORT DOCUMENTATION PAGE

1a. REPORT S	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 93-43	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Naval Medical Command
6c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20889-5055	7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, DC 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20889-5044	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62787A	3M162787	A870.AN1284	DN243540
63807A	3M463807	D808.A01275	DN243520

11. TITLE (Include Security Classification)
Antibodies to the circumsporozoite protein and protective immunity to malaria sporozoites

12. PERSONAL AUTHOR(S) Jones TR; Ballou WR; Hoffman SL

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Book chapter	FROM ______ TO ______	1993	13

16. SUPPLEMENTARY NOTATION
In: Progress in Clinical Parasitology. Vol.III. Editor-in-chief, Tsieh Sun. New York: Springer-Verlag. 1993 pages 103-115

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	malaria; antibody; sporozoite; plasmodium; immunity; circumsporozoite protein

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

93-15272

93 7 02 122

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Librarian	22b. TELEPHONE (Include Area Code) (301) 295-2188 — 22c. OFFICE SYMBOL MRL/NMRI

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

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Antibodies to the Circumsporozoite Protein and Protective Immunity to Malaria Sporozoites

Trevor R. Jones, W. Ripley Ballou, and Stephen L. Hoffman

Malaria is caused by members of the genus *Plasmodium*. The four species infecting humans are *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Plasmodium sporozoites develop in the salivary glands of female anopheline mosquitoes and are transmitted to humans during a blood meal. The sporozoites remain in the host's circulation for a brief period before entering hepatocytes, where they develop for 5–16 days depending on the species. During this period they increase in number thousands of times. A uninucleate *P. falciparum* sporozoite, for example, can develop into a multinucleate liver schizont with as many as 30,000–50,000 uninucleate merozoites. The phase beginning with sporozoite entry into the blood and ending with merozoite invasion of erythrocytes is called the exoerythrocytic phase of the disease.

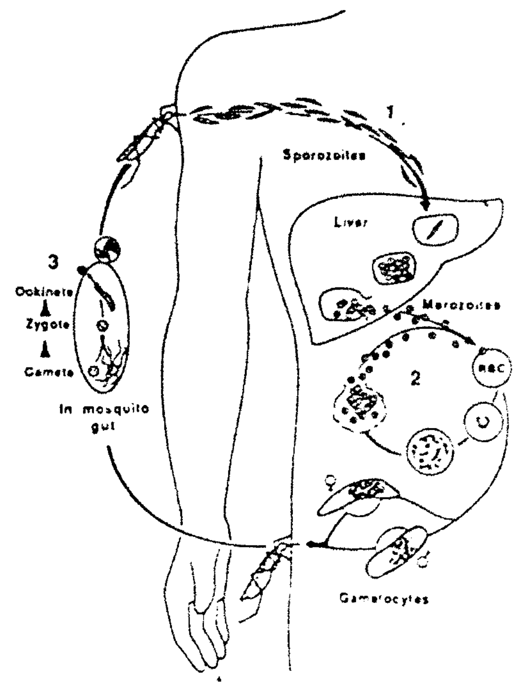

Figure 4.1. Life cycle of the malaria parasite. Sporozoites enter the blood stream when a female anopheline mosquito takes a blood meal (1). The sporozoites enter liver cells and amplify their number thousands of times through the asexual production of merozoites. When the liver cells rupture, merozoites pour into the bloodstream and enter red blood cells (2). Clinical symptoms appear when the mature erythrocytic schizont ruptures. Some of the blood stage parasites differentiate into the sexual stages, gametocytes. When these organisms are taken up by another feeding mosquito, sexual reproduction occurs within the mosquito midgut (3), and sporozoites eventually appear in its salivary glands.

The life cycle of *Plasmodium* reveals several apparent points at which to attack and disrupt the life cycle of the malarial parasite (Fig. 4.1). Investigators are working to develop vaccines that produce immunity to circulating sporozoites, to parasites developing in the liver, to merozoites free in the bloodstream, to parasite-infected erythrocytes, and to the gametocyte. Our efforts are directed at developing vaccines to preerythrocytic stages of the parasite, in particular the sporozoite and the developing liver stage parasite.

Sporozoite Immunity and Circumsporozoite Protein

Studies with avian malarias by Richards et al. (1) indicated that immunization with inactivated sporozoites induced a partial immunity in birds. In 1967 and 1969, Nussenzweig and colleagues (2,3) demon-

strated that mice immunized with radiation-attenuated *P. berghei* sporozoites were protected against challenge with infective, normal *P. berghei* sporozoites. Studies also demonstrated that this sporozoite immunity was stage-specific but not species-specific. In other words, no protection was seen when the immunized mice were challenged with blood stage parasites of *P. berghei*, but protection was seen when mice were challenged with sporozoites from either *P. vinckei* or *P. berghei*. Clyde et al. (4,5) and Rieckmann et al. (6,7) reproduced these studies in humans using irradiated *P. falciparum* and *P. vivax* sporozoites. The important demonstration that humans are biologically capable of generating a protective immune response to malarial sporozoites provided the impetus for subsequent efforts in malaria development. Studies (8,9) have shown that, at least in some strains of mice, the immunity induced by immunization with irradiated sporozoites is mediated by $CD8^+$ T-cells. A large body of evidence already exists, however, indicating that antibody-dependent immunity to sporozoites is possible, and attempts to improve methods of inducing protective antibodies have therefore continued. It now appears that there are two mechanisms of immunity to preerythrocytic stages: T-cell dependent and antibody-dependent. This chapter examines the background and current status of efforts to create vaccines that induce antibodies that prevent effective sporozoite invasion of hepatocytes.

In 1980 an apparent target of the antibody response was localized on the surface of the *P. berghei* sporozoite by Yoshida and coworkers (10). This immunogenic protein, named circumsporozoite (CS) protein, was detected through the use of a monoclonal antibody induced by the bites of irradiated *P. berghei*-infected mosquitoes. In *P. berghei*, the protein has a molecular weight of 44 kDa. Shorthly thereafter, analogous proteins were found on the sporozoite surface of other species of *Plasmodium* (11,12). Within a few years of these observations, the genes for the CS proteins of *P. knowlesi* (13,14), *P. cynomolgi* (15), *P. falciparum* (16,17), *P. vivax* (18,19), *P. berghei* (20,21), *P. yoelii* (22,23), and *P. malariae* (24) were cloned and sequenced. These proteins are generally similar in structure, and all possess a highly immunogenic set of repeated amino acid sequences. The exact amino acid sequence of the repeats varies among species; some repeats are as short as four residues, others as long as twelve (Fig. 4.2).

In 1980 Potocnjak and coworkers (25) made the important observation that passive transfer of antibody alone can provide protection from sporozoite challenge. This antibody reacted with the 44-kDa CS protein that had been described by Yoshida et al. (10). Fab fragments [monovalent immunoglobulin G (IgG) fragments prepared by

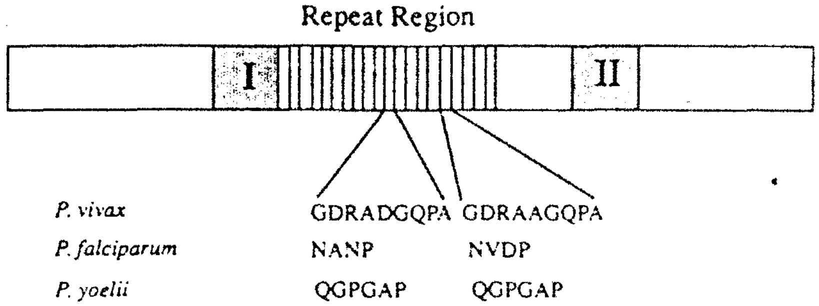

Figure 4.2. Simplified diagram of the *Plasmodium* circumsporozoite protein. Regions I and II represent highly conserved regions with considerable homology between species. The highly immunodominant central repeat region contains repeated amino acid sequences characteristic of each species. Two repeats in the diagram are expanded to show representative sequences from three species. *P. vivax* has 19 copies of a nine amino acid repeat, two of several variant sequences are shown. *P. falciparum* has 23 copies of a four amino acid repeat. NANP is the major repeat, and NVDP is the minor repeat. *P. yoelii* has 15 to 19 copies of a six amino acid repeat (QGPGAP) and six to seven copies of a minor repeat (QQPP).

papain treatment] of this antibody were then successfully used in passive transfer studies in which 10 μg of Fab were intravenously injected into mice that were protected against challenge with 1000 *P. berghei* sporozoites. In comparison studies, the Fab fragments proved as effective as intact antibody at providing protection. Within 5 years of the release of these data, the sequences of the CS proteins of several species of *Plasmodium* were published (vide supra) and a concerted effort began to develop methods of inducing antibody-mediated immunity against sporozoites.

P. falciparum and Human Vaccine Trials

With the identification of the central repeat region of the CS protein of *P. falciparum* in 1984 (16,17), attempts to induce sterilizing immunity to sporozoites began to focus on the use of peptides as immunogens. Young and colleagues (26) transformed *Escherichia coli* with the gene encoding the four amino acid repeat sequence of the *P. falciparum* CS protein; the gene product elicited high antibody titers in mice. Ballou et al. (27) used synthetic peptides based on the *P. falciparum* CS protein conjugated to a carrier to induce

antibodies in mice and rabbits. These antibodies recognized native CS protein and blocked sporozoite invasion of human hepatoma cells. Antibodies raised to two conserved nonrepeat flanking regions (regions I and II) did not neutralize sporozoites. These data were interpreted to mean that the central repeat region was the preferred immunogen for a sporozoite vaccine. Weber and Hockmeyer (28) used the cloned CS protein gene from one strain of *P. falciparum* to probe 17 other *P. falciparum* strains from around the world and found that the probe hybridized with each strain. This finding led them to conclude that the gene was highly conserved and its product could be considered a good candidate for vaccine development. Zavala et al. (29) used sera from a malarious area, monoclonal antibodies to sporozoites, and polyclonal antibodies to $(NANP)_3$ to show that antibody raised against the native immunogens reacted with $(NANP)_3$, and antibody to $(NANP)_3$ reacted with sporozoites. This finding also supported the contention that the central repeat region was an excellent peptide on which to base a sporozoite vaccine. Zavala et al. (30) and Yoshida and colleagues (31) further showed that *P. falciparum* sporozoites collected worldwide reacted with antibody to the repeat region of the CS protein, thereby implying that there was no antigenic variation among strains, and that the repeat region was an excellent target for development as a vaccine.

The first human vaccines against the *P. falciparum* sporozoite were based on the sequence NANP and the minor repeat NVDP. Ballou and colleagues (32) used a recombinant protein vaccine containing copies of both NANP and NVDP plus 32 amino acids from a bacterial tetracycline resistance gene and adsorbed this vaccine to alum ($R32tet_{32}$). Herrington and coworkers (33) used a synthetic peptide vaccine consisting of three copies of NANP conjugated to tetanus toxoid and also adsorbed to alum. In the Ballou study, six subjects immunized with 100-to 800-μg doses were challenged with the bites of five infected mosquitoes; one subject was protected. In the Herrington study, three vaccinated subjects were likewise challenged, and one was protected. In both studies the subjects not infected upon challenge were also the ones with the highest antibody levels, and five of nine of these subjects experienced lengthened prepatency periods that correlated with the antibody titer. The antibody levels induced by these vaccines were low in most volunteers, although the few subjects who responded well developed titers that approached those in persons from malaria-endemic areas. As Hoffman and coworkers (34) demonstrated, however, naturally acquired antibodies to sporozoites seen in persons living in malarious areas do not correlate with resistance to malaria.

Antibody Induction with Subunit Vaccines

The initial human vaccine trials highlighted two issues concerning CS repeat vaccines. The first was the relatively poor antibody response of a significant number of individuals to the vaccines, and the second was the poor efficacy in the presence of what would be considered relatvely high antibody titers for vaccines against viruses or bacteria. During this period, the CS protein of the murine malaria parasite *P. berghei* was cloned and sequenced (20,21). To evaluate the response to subunit CS vaccines, Egan and colleagues (35) immunized mice with a peptide consisting of an eight amino acid repeat sequence found in the CS protein of *P. berghei* (DPAPPNAN conjugated to keyhole limpet hemocyanin). Other mice received an *E. coli* produced recombinant protein containing a large portion of the CS protein including the entire repeat region. By using complete and incomplete Freund's adjuvant, they were able to achieve high levels of antibody, but both vaccines protected only about 50% of mice challenged with a small number of infective sporozoites. Naive mice were then passively immunized with varying amounts of the purified IgG from the immunized mice, and protective efficacy was compared to that achieved with a protective monoclonal antibody. All mice receiving the monoclonal antibody were protected; and 75% were protected with purified polyclonal IgG taken from mice immunized with peptide. These data implied that, on a weight-to-weight basis, polyclonal antibodies appeared at least as effective at providing protection as did monoclonal antibodies. Later, in 1987, Zavala and colleagues (36) obtained even better results by immunizing with a discrete protein. They immunized with a slightly different repeat (DPPPPNPN conjugated to tetanus toxoid) from the *P. berghei* CS protein than the one uses by Egan et al. (35). The vaccinated mice were challenged with a small number (1000) of infective sporozoites. This number is considered a small challenge because the dose of *P. berghei* sporozoites required to infect 50% of mice (ID_{50}) is generally about 500 sporozoites and because *P. berghei* irradiated sporozoites induce an immune state so solid that mice can withstand sporozoite challenges of 50,000–500,000 infective sporozoites (37; S.L. Hoffman and W.R. Ballou; unpublished data). Nevertheless, up to 87% of the mice were protected.

Charoenvit and colleagues (38) showed that antibody reactivity with sporozoite proteins did not imply the ability to protect. Five monoclonal antibodies generated against *P. yoelii* sporozoites were positive for reactivity to *P. yoelii* sporozoites by immunofluorescence; only three were positive in the circumsporozoite precipitation

test. Of these three, only two provided protection when sporozoites were incubated with antibody prior to injection into naive mice (sporozoite neutralization technique). Shortly after this work was published, the sequence of the *P. yoelii* CS protein was determined (22,23) using one of the protective antibodies (NYS1) developed by Charoenvit et al. (38). The protein contained a central repeat region with a dominant repeat having the sequence QGPGAP and the minor repeat QQPP. Incubation with synthetic QGPGAP inhibited the reactivity of the protective antibody NYS1. Sedegah and coworkers (39) used either irradiated *P. yoelii* sporozoites or a vaccinia recombinant construct encoding the full-length *P. yoelii* CS protein including 19 copies of QGPGAP to vaccinate mice. Both groups of animals generated excellent antibodies against the $(QGPGAP)_2$; the irradiated sporozoite vaccinated group was protected upon challenge with 10,000 infective sporozoites, the recombinant construct vaccinated group was not protected against challenge with 200 sporozoites. However, when the protected mice were depleted of $CD8^+$ T-cells, protection was lost, indicating that neither group generated protective antibodies. Charoenvit and colleagues (40) showed that mice that received NYS1, a monoclonal antibody to the *P. yoelii* CS protein repeat region, were protected upon subsequent sporozoite challenge. However, mice immunized with a *P. yoelii* repeat region subunit vaccine were not protected. Upon examination of the sera from the actively immunized, passively immunized (NYS1 antibody), and irradiated sporozoite immunized mice, no significant difference between the three types of sera was found by the enzyme-linked immunosorbent assay (ELISA) against QGPGAP or immunofluorescence against sporozoites.

These studies, viewed collectively, demonstrate that (1) circulating antibodies alone (in the form of injected monoclonal antibodies) provide potent protection against sporozoite challenge; and (2) polyclonal antisera generated by vaccination with a protein containing the target of the protective antibody provided only partial or no antibody-mediated protection, and the mice remained susceptible to sporozoite challenge. These experiments left two important questions unanswered. Can peptide vaccines induce a protective monoclonal antibody? (All protective monoclonal antibodies to date had been raised by immunization with irradiated sporozoites.) Can peptide vaccines induce a solid, protective polyclonal response as effective as that induced by passive immunization with monoclonal antibodies or active immunization with irradiated sporozoites?

Antibody-Mediated Immunity and Specific Epitopes

Ak and coworkers (manuscript in preparation) used a *P. yoelii* repeat region subunit vaccine to immunize mice and make monoclonal antibodies. Two of the antibodies, an IgG1 and an IgG2b, provide protection upon passive transfer. These findings show that the immune system, at least in mice, is capable of recognizing a small peptide sequence and generating a monoclonal antibody against it that is protective. The issue of whether antibody-mediated protection is IgG subclass-dependent was also put to rest. These data, in combination with the Fab studies of Potocnjak and colleagues (25), show that IgG subclass is not a restricting factor in the ability to provide protection. In the *P. yoelii* system, IgG1, IgG2b, and IgG3 monoclonal antibodies have provided protection (NYS1, a protective monoclonal antibody is an IgG3). In 1984 Charoenvit and colleagues (unpublished data) generated a monoclonal antibody by immunizing mice with irradiated *P. vivax* sporozoites. This antibody, designated NVS3, was used in the cloning and sequencing of the *P. vivax* CS protein, and it binds to the repeat region (DRA A/D GQPAG) of that protein (19). Work by Charoenvit and coworkers (41) demonstrated that when 2 mg of NVS3 were infused into *Saimiri* monkeys, four of six of these monkeys were protected against a 10,000 *P. vivax* sporozoite challenge. This study was the first demonstration that sporozoites from a human malarial parasite could be neutralized solely by circulating antibodies. NVS3 was then subjected to analysis by epitope mapping (42), which required synthesis of many eight amino acid subsets of the repeat region of the *P. vivax* CS protein and showed clearly that the epitope of NVS3 was the four amino acid sequence AGDR (41). In an earlier study performed by Collins et al. (43), *Saimiri* monkeys were immunized with a recombinant subunit vaccine designated $NS1_{81}V20$. This vaccine contains multiple copies of the repeat sequence of the *P. vivax* CS protein and of AGDR. The monkeys immunized with $NS1_{81}V20$ were not protected upon challenge with 10,000 *P. vivax* sporozoites. Subsequent examination of the sera from these monkeys (41) indicated that although they generated high antibody titers to *P. vivax* sporozoites by immunofluorescence and to $NS1_{81}V20$ in ELISA none of the animals produced detectable antibody to $(AGDR)_2$, as determined in ELISA even though multiple copies of AGDR are contained within the $NS1_{81}V20$ sequence. This finding means that even if the desired protective epitope is present in the sequence of the immunogen, the desired antibody response is not ensured. Extraneous amino acids that form nonprotective but apparently immunodomi-

nant epitopes may have to be deleted from the immunogen. These studies demonstrated that great exactitude is required in the selection of the epitope used to induce a protective antibody response. Here an immunogen that is bound by a monoclonal antibody known to be protective in passive transfer studies generated an excellent antibody titer against sporozoites and against itself but failed to produce antibody to the discrete, protective epitope contained within its sequence.

The promise of the AGDR epitope is tempered, however, when one considers data on strain variation in the CS protein repeat region of *P. vivax*. Zavala and coworkers (30) studied seven strains of *P. vivax* from around the world and found that monoclonal antibodies to the repeat region reacted with sporozoites from all seven strains. This nonvariability was consistent with that found in *P. falciparum*. In 1989, however, Rosenberg and colleagues (44) found that more than 14% of the uncomplicated cases of *P. vivax* malaria at two sites in Thailand were caused by a strain with a nine amino acid repeat that shared only a three amino acid homology with the previously published, nonvariant sequences. Antibody reactivity to the variant repeat in persons from *P. vivax* endemic areas has since been reported (45,46).

This variant sequence does not contain AGDR. The possibility, however, that AGDR can be used to immunize and provide protection against nonvariant strains of *P. vivax* means that it may be a paradigm for the development of synthetic or recombinant vaccines against a variety of CS protein repeats in both *P. vivax* and other species of *Plasmodium*.

Carriers and Adjuvants

Concurrent with advances in understanding the crucial importance of epitope specificity (vide supra), methods for significantly improving the antibody titers induced by subunit vaccines are also being developed. Changes in both carriers and the adjuvants have resulted in dramatic improvements in antibody response. J.C. Sadoff and colleagues (Walter Reed Army Institute of Research, personal communication) chemically conjugated R32 (32 copies of the *P. falciparum* repeats) to a variety of protein carrier molecules including tetanus toxoid, choleragenoid, meningiococcal outer membrane protein, diphtheria, and the exotoxin A of *Pseudomonas aeruginosa* (ToxA). All were safe when used in humans, but the R32ToxA construct was the superior immunogen, elicting levels of antibodies in nearly all volunteers equal to those found in the protected volunteers in the Ballou

and Herrington studies (32,33). In addition to improved carriers, new and more effective adjuvants are also now available. Using R32NS1$_{81}$ (R32 plus 81 amino acids from nonstructural protein 1 of influenza A) as the vaccine/carrier, Rickman et al. (47) have shown that the use of a novel adjuvant consisting of detoxified lipid A (MPL) and mycobacterial cell wall skeleton (CWS) in squalane (Detox, Ribi Immunochem) results in as much as a 10-fold increase in immunogenicity compared to R32NS1$_{81}$ adsorbed to alum, the traditional adjuvant. Similar results have been observed when the R32NS1$_{81}$ antigen was incorporated into liposomes containing detoxified lipid A (C. Alving and L. Fries, personal communication). Efficacy studies using these new carrier/adjuvant combinations are currently under way.

Longevity of Antibody

For vaccines designed to induce antibody-mediated protection, the longevity of antibody levels to specific, protective epitopes on the malaria parasite is an important factor when determining the duration of protection. Persons such as travelers who pass through malarious areas require protection for only a specified period of time, whereas persons living permanently in endemic areas need protective antibody levels for life. Antibody titers induced by R32 vaccine candidates combined with both alum and MPL/CWS adjuvants generally drop to 50% with 6 months of the final vaccination (32,47). The effect of this drop on efficacy is unknown. There are several possible ways to extend antibody longevity. Adjuvants or other drug delivery methods may be developed that lead to the continuous production of specific antibody over long periods of time. Another approach would be to include a T-helper epitope in the vaccine. Persons living in endemic areas may receive natural T-helper cell-mediated boosting owing to exposure to the bites of infected mosquitoes. In rodents, there is evidence that sporozoites can boost the level of antibody to subunit vaccines (48), but whether natural exposure can boost or even help maintain antibody levels in humans is not known.

Antibody-Mediated Passive Immunization

A variety of persons including tourists, diplomats, businessmen, and military personnel may receive effective levels of protection from passively transferred antibody. Studies with rodent and human malarias, already described (25,41), demonstrated that circulating antibodies alone can induce protection. The technology exists to convert

urine monoclonal antibodies to chimeric human immunoglobulin 9), thereby reducing reactivity due to species differences. This reacon may provide an avenue for the production of injectable antibody pable of providing short-term protection to those briefly exposed to alaria. In another approach, lymphocytes from appropriately immuzed humans are fused with myeloma cells to make a hybridoma at produces fully human monoclonal antibody. It is also possible to oduce vast numbers of different Fab fragments by expression in *coli* and screen them for reactivity to any desired epitope (50). Once e desired Fab fragment is identified, it can be produced in great iantity. Because Fab fragments have been shown to be protective, at ast in mice (25), it is conceivable that they could then be used for ıssive immunization.

)verview

oday's efforts to develop a sporozoite vaccine were started with the oservation that irradiated sporozoites induced protection in rodents. 'ith the identification and sequencing of the circumsporozoite proin, attention focused on the creation of specific synthetic or recomnant subunit molecules that would induce a potent immunity to orozoites. Passive transfer experiments using monoclonal antiodies generated against both irradiated sporozoites and peptides deonstrate that impressive levels of protection against sporozoite iallenge can be achieved with circulating antibodies alone. The next ep, that of obtaining similar antibody-mediated protection through ımunization with subunit vaccines, has not been reached. Informaon obtained from both *P. vivax* and *P. yoelii* studies, however, indites that the successful induction of protection with monoclonal ıtibodies depends on the exact specificity of the antibody. The probms encountered in inducing polyclonal antibody-mediated protecon that is equivalent to passive immunization with protective onoclonal antibodies suggests that polyclonal responses are perhaps o diffused and of too low titer to neutralize the sporozoite.

Two important factors in the induction of antibody-mediated sporoite immunity have emerged. First, methods for inducing and aintaining high levels of antibodies must be developed. MPL/CWS juvants and liposomes have already been shown to induce those gh levels. Methods for increasing the longevity of antibody, howev, have yet to be developed. Second, it may be ideal for the imunogen to contain only epitope (s) proved to be protective when und by specific antibody. These epitopes can probably be best idenied by the generation of monoclonal antibodies to promising epi-

topes and subsequent passive transfer studies. Preparing an epitope-carrier-adjuvant combination that induces high titer antibodies with extended longevity and correct specificity is now the focus of interest for many malaria vaccine developers.

Acknowledgments

This study was supported by Naval Medical Research and Development Command work unit numbers 3M16102BS13AK111, 3M162787AB70AN121, and 3M463807D808AQ133. Those experiments reported herein that were performed by Department of Defense personnel were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, DHHS Publication (NIH) 86-23, (1985). The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Army Department.

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